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In utero exposure to low doses of environmental pollutants disrupts fetal ovarian development in sheep

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Epidemiological studies of the impact of environmental chemicals on reproductive health demonstrate consequences of exposure but establishing causative links requires animal models using 'real life' *in utero* exposures. We aimed to determine whether prolonged, low-dose, exposure of pregnant sheep to a mixture of environmental chemicals affects fetal ovarian development. Exposure of treated ewes ($n = 7$) to pollutants was maximized by surface application of processed sewage sludge to pasture. Control ewes ($n = 10$) were reared on pasture treated with inorganic fertilizer. Ovaries and blood were collected from fetuses ($n = 15$ control and $n = 8$ treated) on Day 110 of gestation for investigation of fetal endocrinology, ovarian follicle/oocyte numbers and ovarian proteome. Treated fetuses were 14% lighter than controls but fetal ovary weights were unchanged. Prolactin (48% lower) was the only measured hormone significantly affected by treatment. Treatment reduced numbers of growth differentiation factor (GDF9) and induced myeloid leukaemia cell differentiation protein (MCL1) positive oocytes by 25–26% and increased pro-apoptotic BAX by 65% and 42% of protein spots in the treated ovarian proteome were differently expressed compared with controls. Nineteen spots were identified and included proteins involved in gene expression/transcription, protein synthesis, phosphorylation and receptor activity. Fetal exposure to environmental chemicals, via the mother, significantly perturbs fetal ovarian development. If such effects are replicated in humans, premature menopause could be an outcome.

Keywords: environmental chemicals; fetal development; oocyte; granulosa cell; sewage sludge

Introduction

Exposure to environmental compounds (ECs) may alter female reproductive tissues and thus affect the ability of human couples to conceive and maintain a healthy pregnancy (Hruska *et al*., 2000). ECs include endocrine disrupting compounds [EDCs, e.g. dioxins, polychlorinated biphenyls (PCB) and organochlorine pesticides] that have been/are used extensively in manufacturing and agriculture. ECs are found ubiquitously within the environment, together with heavy metal pollutants. Human exposure occurs through a variety of routes including the consumption of meat and dairy products, ingestion of water, absorption through the skin and inhalation. The concentrations of ECs within human tissues and the mechanisms through which they elicit effects on reproductive tissues are not well understood. Studies conducted in a variety of animal models demonstrate that the female fetus is particularly sensitive to small endocrine changes (Lovekamp-Swan and Davis, 2003; Henley and Korach, 2006) that have detrimental effects on reproductive function (Miller *et al*., 2004; Uzumcu and Zachow, 2007). There is uncertainty as to whether women's fertility and reproductive health have deteriorated or whether the link between exposure to ECs and increased incidence of breast cancer, precocious puberty, premature menopause and decreased fertility is valid (Sharara *et al*., 1998; Hruska *et al*., 2000). Nevertheless, the importance of developmental disturbance in disease aetiology is increasingly evident (Heindel, 2006). Determining the incidence of reproductive problems in women (15–20% of couples have difficulty conceiving) is confounded by diagnostic, social (e.g. increasing age of women first trying to conceive) and obesity issues

(Evers, 2002; Sharpe and Franks, 2002) which complicates linking changes in reproductive function with EC exposure (Akkina *et al*., 2004; Cesario and Hughes, 2007). Similarly, although the incidence of breast cancer is clearly rising and has a strong links with environmental factors and *in utero* exposures (Kortenkamp, 2006), there is no general increase in ovarian cancer (Bray *et al*., 2005). Two conclusions arise from the literature: first, that the development of the female fetus or embryo can be perturbed by exposure to ECs and, second, that the evidence for population trends in humans is mixed and often inconclusive.

Human tissues, including the maternal-fetal unit and maternal tissues during gestation, contain levels of ECs that are associated with many *in utero* effects (e.g. Ikezuki *et al*., 2002; Younglai *et al*., 2002; Tsutsumi, 2005; Barr *et al*., 2007; Chao *et al*., 2007; Huang *et al*., 2007; Thundiyil *et al*., 2007). However, the lack of robust human exposure-phenotype linkage data and multi-factorial effects on reproductive trends hampers our ability to test the hypothesis that *in utero* exposure to environmental chemicals damages female reproductive development (Foster, 2003). Many ECs have the potential to perturb development (Tabb and Blumberg, 2006; Watson *et al*., 2007) and epidemiological studies (Toft *et al*., 2004) suggest that there is a need to study the effects of EC exposure in relevant animal models at real-world rates of exposure, focusing on likely 'sensitive' components and mechanisms of relevant reproductive circuits. Most studies into effects of ECs have focused on rodent models, using single compounds, administered for short periods, often at pharmacological doses. Although essential to understanding of the mechanisms though which such compounds act, these studies are of limited value in the assessment of risks to human developmental health because the patterns of experimental exposure are not representative of normal human exposure. Thus an animal model using a prolonged, 'real-life' (defined as exposure through natural routes, not via specific application, such as injection), in-utero, pattern of EC exposure will be better able to indicate likely effects on humans.

An ovine model in which pregnant ewes are exposed to the complex mix of ECs and heavy metals contained in human sewage sludge, following its application to pasture as a fertilizer, is characterized by defects in fetal development (Paul *et al*., 2005). To determine which chemical classes might be involved in these effects, we measured levels of seven heavy metals, the di(2-ethylhexyl) phthalate (DEHP), nonyl phenol and seven polychlorinated biphenyl (PCB) congeners in tissues of exposed fetuses/offspring and found concentrations of Cu, Pb, Zn and DEHP to be significantly altered (Rhind *et al*., 2002, 2005a,b, 2007a,b). Sewage sludge is a relevant source of a chemical cocktail to which humans are exposed as it, in itself, broadly reflects human exposure (Rhind *et al*., 2002, 2005b; Abad *et al*., 2005; Oleszczuk, 2006; Martinez *et al*., 2007) to a complex mixtures of ECs (Groten *et al*., 2004; Jonker *et al*., 2004; Robinson and MacDonell, 2004; Koppe *et al*., 2006). In addition, sheep, like humans, are long-lived and have a relatively long gestation period (145 days), during which time ovine fetuses show a similar timing and sequence of ovarian development to that observed in the human (Pryse-Davies and Dewhurst, 1971; Juengel *et al*., 2002; Sawyer *et al*., 2002; De Felici *et al*., 2005). Unlike the rodent, the ruminant fetal ovary synthesizes estrogens which are important for germ cell development (Pannetier *et al*., 2006). Therefore, notwithstanding the metabolic and dietary differences between sheep and humans, the long-term, real-life, low-dose, exposure of pregnant sheep to a complex mix of relevant ECs is a good model for the human given that we know that many ECs are gaining access to the materno-fetal unit in pregnant women.

In this study, we aimed to determine whether prolonged, low-dose, exposure of the developing fetus, *in utero*, to maternal dietary EC load adversely affects the developing fetal ovary.

Materials and Methods

Animals, blood and tissue collection

The study was conducted at the Macaulay Institute research station (Hartwood, Lanarkshire, Scotland, UK) using Texel ewes. Animals were maintained on pasture at conventional stocking rates, adjusted according to pasture height, as described previously (Paul *et al*., 2005), so that animals from the respective treatments were maintained in comparable nutritional states. Neither group received supplementary feeding during their breeding lives. The animals were inspected by a qualified shepherd on a daily basis and routine animal care and vaccination procedures were conducted, as prescribed by best practice protocols.

Digested sewage sludge was applied twice annually, to each of three replicate 9 ha plots, at a rate of 2.25 metric tons of dry matter per hectare. For the first five applications, the sludge was applied in liquid form as described previously (Rhind *et al*., 2002). Thereafter, owing to changes in sludge production practices by the UK water authorities, thermally dried sludge pellets were used and applied at similar rates (about 2.25 tones dry matter/ha/year). The composition of the sludge, on a dry matter basis, did not differ between the two methods of application. The application of sludge to the surface of the pastures was not designed to conform to the UK recommendations for good practice (SEDE and Arthur Anderson, 2001). According to recognized codes of practice, sludge can only be applied to grazed grassland when it is deep injected into the soil or, if it is applied to the surface, there can be no grazing of that land within the season of application. However, this study was designed to maximize the rate of contamination of the pasture and topsoil and thus to maximize the risk of exposure of grazing animals to the chemical constituents of sewage sludge through their food'. Animals were not allowed to graze the pasture until a minimum of 3 weeks after sludge application, as prescribed by relevant legislation (Parliament, 1989). Control ewes were maintained on similar pasture to which 225 kg of nitrogen/ha/year was applied in the form of conventional inorganic fertilizers. The relatively harsh environmental conditions in central Scotland where the farm is located mean that there was no growth of clover or other estrogenic plant species in any of the pastures.

The treated and control groups from which the study animals were drawn each comprised 3 replicate subgroups of 5 breeding ewes in each of four age categories (total flock size = 120 ewes). For the present study, subgroups of ewes that were 6 years of age and had been maintained on the respective treatments throughout their breeding lives were drawn from all replicates of each treatment. Ewes from both groups underwent estrous cycle synchronization, using progestagen sponges (Chronolone, 30 mg; Intervet, Cambridge, UK), before being mated to rams of the same genotype and from the same source, to remove the effect of genotypic differences. Estimation of gestational age was based on the knowledge that conception should occur within 48 h of sponge removal when estrus cycle synchronization is used. Animals were euthanized at ~110 days of gestation (GD110, equivalent to 27 weeks in the human) according to Schedule 1 protocols as defined by the UK Animals (Scientific Procedures) Act, 1986. Twenty-three female and 24 male fetuses were collected from both sets of ewes (control, $n = 10$, treatment, $n = 7$) and all of the resulting female fetal lambs ($n = 15$ in control, $n = 8$ in treated groups) were used for the ovary studies detailed below. Maternal and fetal body weight and fetal ovary weight were recorded at slaughter; fetal blood samples were also collected and the serum, isolated by centrifugation, stored at −20°C until required for assay. One ovary from each animal was fixed for 5.5 h in Bouin's fixative and then transferred to 70% ethanol until analysis. The other ovary from each animal was snap-frozen in liquid N_2 and stored at −80°C until analysis. GD110 was selected as a representative developmental stage since the primordial follicle pool has been established and expression of many developmentally important genes, such as growth differentiation factor 9 (GDF9) is maximal (Mandon-Pepin *et al*., 2003).

Immunohistochemistry

Sagittal 5 μm sections of each fetal ovary were cut, floated onto slides and dried at 50°C overnight. Slides were de-waxed in xylene, hydrated gradually through graded alcohols and washed in water. For all ovaries, slides were prepared containing two randomly selected, non-consecutive sections. At least one slide from each ovary was H&E stained for morphological assessment. Further slides were immunostained for induced myeloid leukaemia cell differentiation protein (MCL1) (pro-proliferative), GDF9 (development of primordial

follicles) and phosphorylated histone H3 (pH3), to assess active proliferation and establish a mitotic index (Brenner *et al*., 2003). Following routine de-waxing, antigen retrieval procedures were used for the MCL1 and GDF9 epitopes [microwaving in 0.01 M citrate buffer (pH 6.0) on full power for 3 × 5 min]. Immunostaining for MCL1 and GDF9 was carried out using standard peroxidase-based immunostaining protocols (MCL1: chemMate, Dako and GDF9: vector). Slides for MCL1 were placed in a Dako autostainer and incubated with anti-MCL1 antibody (Serotec, 1/50), for 30 min. Antibody binding was visualized using the ChemMate peroxidase/DAB detection system (DakoCytomation Ltd., Ely, Cambridgeshire, UK). Slides for GDF9 were incubated overnight in a humid chamber with anti-GDF9 antibody (R & D Systems, 1/15) and antibody binding visualized using the Vector peroxidase/DAB detection system for 30 min (Vector Laboratories, Peterborough, UK). A rabbit anti-P-Histone H3 (#06–570; 1:500; Upstate Cell Signalling Solutions, Hampshire UK) was used after antigen retrieval and visualized using a goat biotinylated anti-rabbit antibody, followed by AB complex with horseradish peroxidase (HRP) and DAB detection (all DAKO, Cambridge, UK). Ovarian sections were analysed for oocyte numbers and follicle size classes using an established follicle classification system (Lundy *et al*., 1999) by two independent observers (N.J.D. and H.M.) using six fields of view per section. Total numbers of pH3-positive granulosa, stromal, endothelial and surface epithelial cells were counted at ×40 and identified at ×400 by M.A. Because of the relatively low incidence of pH3-positive cells, cell numbers were expressed per mm^2 of ×40 fields of view. In all analyses, sections from 15 control and 8 sewage sludge exposed fetuses were used. Only oocytes with the nucleus clearly visible were included in quantification of oocyte and follicle densities, which was performed in six separate slides containing a total of 12 sections for each fetus (H&E, MCL1 and GDF9).

Protein extraction

The frozen fetal ovaries were processed for one-dimensional and two-dimensional gel electrophoresis, as described in detail previously (Fowler *et al*., 2007a,b). Briefly, the frozen ovaries (15 control, 8 treated) were blotted on filter paper and combined with lysis buffer (1 mg wet tissue weight: 5 μl lysis buffer) containing 0.01 M Tris–HCl, pH 7.4, 1 mM EDTA, 8 M Urea, 0.05 M DTT, 10% (v/v) glycerol 5% (v/v) NP40, 6% (w/v) pH 3–10 Resolyte (Merk Eurolab Ltd, Poole, Dorset, UK) and protease inhibitor cocktail (Roche Diagnostics, Lewes, UK). The tissues were minced, sonicated in iced water for four 10 min bursts, with 2 min between each sonication, and centrifuged at 50 000*g* for 20 min at 4°C. Once the protein content of the final supernatant containing the soluble cellular proteins had been determined (RC-DC assay, Bio-Rad Laboratories Ltd, Hemel Hempstead, UK), the ovary extracts were stored at −80°C until required for further analysis.

One-dimensional gel electrophoresis and western blot

Individual ovary lysates (15 control and 8 treated) were electrophoresed (30 μg protein/lane) on 26-lane one-dimensional gel electrophoresis 4–12% Bis–Tris gels (Invitrogen Ltd, Paisley, UK) under reducing conditions and transferred to immobilon-FL membrane [Millipore (UK) Ltd, Watford, UK] as described previously (Lea *et al*., 2005). SeeBlue plus two molecular weight markers (Invitrogen) were electrophoresed in three lanes of every gel. The membranes were blocked (overnight, 4°C) with (Odyssey Blocking Buffer, 927–4000: LI-COR Biosciences UK Ltd, Cambridge, UK) and were incubated with primary antibodies (in blocking buffer) at 4°C overnight: (i) BAX (1:200: santa-Cruz Biotechnology Inc, CA, USA, sc-493), (ii) BCL2 (1:200: santa-Cruz, sc-783), (iii) SCF (1:200: santa-Cruz, sc-113126), (iv) CYP17A1 (1:2500: Fowler *et al*., 2007a), (v) SOD2 (1;2500: abnova Corp, Taipei, Taiwan, H00006648), all combined with an anti-β-actin load control of differing species (mouse 1:5000 AB6276; rabbit 1:10 000 AB8227, both AbCam Ltd, Cambridge, UK). The protein bands were visualized using an Odyssey infrared fluorescence imager (LI-COR) and the resulting electronic images were analysed using Phoretix-1D Advanced software (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK) in order to determine the band volumes and molecular weights. This software calculates band volumes, based on constant lane width and automatic band selection, from the raw data of pixel area and intensity that are independent of operator-altered contrast or brightness. The band volumes of β-actin were compared between groups to check the validity of this load control for ovaries from control and treated groups.

Proteomics

In the present study, we have selected proteomic methods as an exploratory tool to uncover novel effects on the ovary; we have previously demonstrated the utility of this approach for heterogeneous tissues (Fowler *et al*., 2007a,b) and others have used it to characterize responses to endocrine disruption (Alm *et al*., 2006).

Two-dimensional gel electrophoresis

Soluble proteins were analysed by two-dimensional gel electrophoresis using a small format gel system described previously (Cash and Kroll, 2003; Uwins *et al*., 2006), using pooled (control versus sewage sludge) lysates comprising equal quantities of protein from each ovary (15 ovaries in the control pool, 8 ovaries in the treated pool). Briefly, in order to ensure the clearest electrophoresis of the protein by two-dimensional gel electrophoresis, sample clean-up was performed using ReadyPrep 2-D Cleanup Kits (Bio-Rad Laboratories Ltd, Hemel Hempstead, Herts, UK) according to manufacturer's instructions. Hundred microgram of total protein from the pooled lysates were separated in the first dimension on 7 cm pH 4–7 immobilized pH gradient gels (GE Healthcare, Uppsala, Sweden). For the second dimension, the proteins were resolved on 10–15% gradient polyacrylamide slab gels and proteins were detected using Colloidal Coomassie Blue G250. Four replicate gels were electrophoresed from each lysate pool. Stained gels were scanned using a Molecular Dynamics Personal Densitometer (GE Healthcare) at 50 μm resolution to generate 12-bit images, which were transferred to Phoretix 2D Analytical software, V 6.01 (Nonlinear Dynamics, Newcastle, UK). The semi-automated routines available in this software were used to detect and quantify protein spots as well as to match the profiles across a gel series. Individual spot volumes are expressed as normalized volumes relative to the total detected spot volume separately for each gel, minimizing potential analytical artefacts from variations in protein loading and migration.

Mass spectroscopic protein identification

Proteins in the gel pieces were digested with trypsin (sequencing grade, modified; Promega UK, Southampton, UK) using an Investigator ProGest robotic workstation (Genomic Solutions Ltd., Huntingdon, UK). Briefly, proteins were reduced with DTT (60°C, 20 min), S-alkylated with Iodoacetamide (25°C, 10 min) then digested with trypsin (37°C, 8 h). The resulting tryptic peptide extract was dried by rotary evaporation (SC110 Speedvac; Savant Instruments, Holbrook, NY, USA) and dissolved in 0.1% formic acid for LC-MS/MS analysis. Peptide solutions were analysed using an HCTultra PTM Discovery System (Bruker Daltonics Ltd., Coventry, UK) coupled to an UltiMate 3000 LC System [Dionex (UK) Ltd., Camberley, Surrey, UK]. Peptides were separated on a Monolithic Capillary Column (200 μm i.d. × 5 cm; Dionex part no. 161409). Eluent A was 3% acetonitrile in water containing 0.05% formic acid, Eluent B was 80% acetonitrile in water containing 0.04% formic acid with a gradient of 3–45% B in 12 min at a flow rate of 2.5 μl/min. Peptide fragment mass spectra were acquired in data-dependent AutoMS(2) mode with a scan range of 300–1500 m/z, three averages, and up to three precursor ions selected from the MS scan 100–2200 m/z. Precursors were actively excluded within a 1.0 min window, and all singly charged ions were excluded. Peptide peaks were detected and deconvoluted automatically using Data Analysis software (Bruker). Mass lists in the form of Mascot Generic Files were created automatically and used as the input for Mascot MS/MS Ions searches of the NCBInr database using the Matrix Science web server (www.matrixscience.com). The default search parameters used were: enzyme = Trypsin; Max. Missed cleavages = 1; Fixed modifications = Carbamidomethyl (C); Variable modifications = Oxidation (M); Peptide tolerance ± 1.5 Da; MS/MS tolerance ± 0.5 Da; Peptide charge = 2+ and 3+; Instrument = ESI-TRAP. Only proteins showing good agreement with mass and pI on the two-dimensional gels, statistically significant MOWSE scores and good sequence coverage were considered to be positive identifications.

Hormone assays

Fetal serum levels of follicle-stimulating hormone (FSH), estradiol and prolactin (PRL) were measured by radioimmunoassays that have been described and validated previously for sheep (McNeilly *et al*., 1986; Mann and Lamming,

Table I. Effects of sewage sludge on morphological and endocrine characteristics of Day 110 fetal ewes.

Characteristic	Control $n = 15$	Sludge-exposed $n = 8$	Fold-change following sludge-exposure	ANOVA
Body weight (g)	1593 ± 67	1365 ± 67	−1.17	$P = 0.013$
Ovary weight (mg)	29.4 ± 2.1	31.0 ± 4.2	+1.05	NS
FSH (ng/ml)	2.1 ± 0.3	1.4 ± 0.2	−1.50	NS
Estradiol (pmol/l)	128 ± 54	100 ± 27	−1.28	NS
PRL (ng/ml)	3.7 ± 0.7	1.9 ± 0.3	−1.95	$P = 0.011$

Lack of statistical significance (NS) is indicated where $P > 0.05$.

1995; Lincoln *et al*., 2003; Crawford *et al*., 2004). The assay standards used, assay sensitivities and intra-assay coefficients of variation were: FSH; NIDDK-FSH-RP2 and NIH-LS18, 0.1 ng/ml, <10%, estradiol; MAIA estradiol kit (Serono Diagnostics, Fleet, Hants, UK), 0.2 pg/ml, <12%, PRL; NIDDK-PRL—RP3, 0.5 ng/ml, 10%). All fetal blood samples were measured in single assays.

Statistical analysis

Analyses were performed using JMP (5.1, Thomson Learning, London, UK). Normality of data distribution was tested with the Shapiro–Wilk test and non-normally distributed data were log-transformed prior to analysis. Morphological and endocrine data and the one-dimensional gel electrophoresis WB band volumes (normalized relative to β-actin expression separate for each lane) and normalized spot volumes (% of total spot volume for each gel separately) were compared as control versus sewage sludge-exposed using one-way ANOVA. For the proteomics, virtual 'average' gels were prepared so that only spots present in three out of four gels for each group were included and compared to determine differences in spot expression. Spots demonstrating statistically significant differences (ANOVA) in normalized volume were investigated further. Unless stated, otherwise data are presented as mean ± SEM.

Results

Maternal and fetal body weights and fetal ovary weights and endocrinology

There was no difference in the body weights, at the time of slaughter, of the ewes pastured on control and sewage sludge-treated fields (83 ± 2 versus 84 ± 1 kg, respectively). In contrast, the female fetuses from the ewes maintained on the sewage-treated pastures were significantly ($P = 0.013$) lighter (14%) (Table I) than the controls [and similar to the male fetuses (Paul *et al*., 2005)]. Despite difference in body weight, there was no significant ($P > 0.05$) treatment effect on the ovary weights. When ovary weights were normalized to body weight (mg/kg), the difference between treated and control animals remained non-significant (treated 22.7 ± 3.0 versus control 18.8 ± 1.1 mg/kg, $P > 0.05$). Of the three hormones measured, only PRL was significantly affected by exposure to sewage sludge (reduced 49% compared with controls, $P = 0.011$) although estradiol concentrations tended to be lower (22%) than the controls.

Effects of treatment on oocyte and follicle numbers and mitotic index

The densities of oocytes and follicles were similar, whether quantified by assessing H&E stained slides or MCL1 and GDF9 positive oocytes (Fig. 1a and b). Total oocyte densities were significantly reduced in the treated ovaries assessed by H&E staining (19% reduced: 18.8 ± 1.3 in treated versus 23.1 ± 1.6 in control, $P = 0.042$), MCL1 immunostaining (26% reduced: 14.6 ± 2.1 in treated versus 19.6 ± 1.5 in control, $P = 0.039$, Fig. 1) and GDF9 immunostaining (28% reduced: 13.9 ± 1.9 in treated versus 19.4 ± 1.4 in control, $P = 0.034$). The ratio of different follicle classes and isolated oocytes was also significantly skewed ($P = 0.003$ by two-way ANOVA combining treatment with

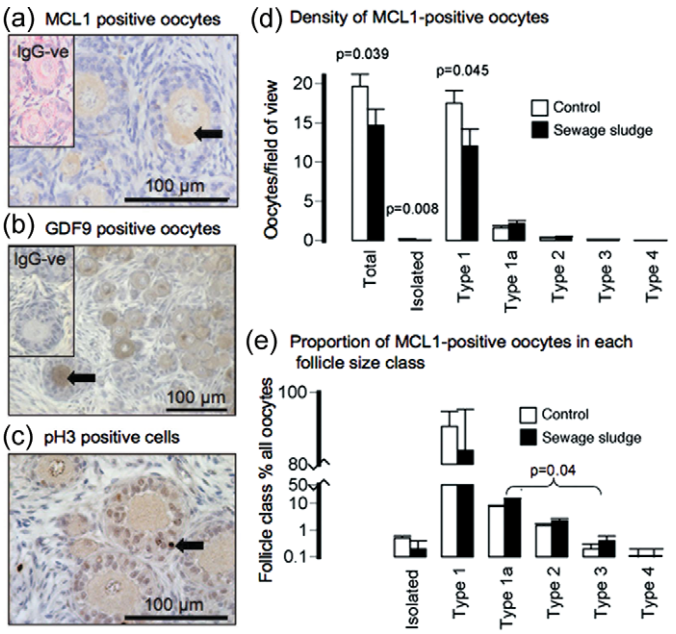

Figure 1: Effect of sewage sludge exposure on fetal ovarian morphology. Immunolocalization of (**a**) MCL1, (**b**) GDF9 and (**c**) phosphorylated histone H3 expression in representative Day 110 fetal ovary sections. In (a) and (b), the arrow highlights immunopositive oocytes, whereas in (c), the arrow shows a histone pH3 positive granulosa cell. GDF9 and MCL1 immunopositive oocytes were present in all classes of follicles observed. Quantification ($n =$ 15 control versus 8 treated ovaries) of the MCL1 immunohistochemistry is shown in (**d**) demonstrating the significant decrease in oocyte density in sewage sludge-exposed fetuses and (**e**) demonstrating the small but significant increase in the proportion of more advanced follicles in sewage sludge-exposed fetuses. Since the findings were similar between H&E staining and MCL1 and GDF9 positive oocytes, only the MCL1 data are shown to illustrate these results.

follicle size class) by treatment (Fig. 1e) with a slightly, but significantly, greater proportion of the follicles being further developed following EC exposure: (combining classes 1a, 2 and 3: treated 16.1 ± 1.8 versus control 11.2 ± 0.9, $P = 0.040$). There were no statistically significant effects of sewage sludge exposure ($P > 0.05$) on the mitotic index of granulosa cells (0.6 ± 0.3 in treated versus 1.0 ± 0.2 in control), stromal cells (14.1 ± 1.3 in treated versus 13.3 ± 1.0 in control) or surface epithelial cells (0.3 ± 0.2 in treated versus 0.5 ± 0.1 in control), expressed as pH3-positive cells/mm^2. In contrast, sewage sludge-exposed ovaries had significantly ($P = 0.014$) more pH3-positive endothelial cells/mm^2 (1.2 ± 0.3 treated versus 0.3 ± 0.2 control).

Effects of treatment of markers of apoptosis, developmental signalling, steroidogenesis and response to oxidative stress

There were no significant differences in β-actin band volumes between treated and control groups, demonstrating the validity of

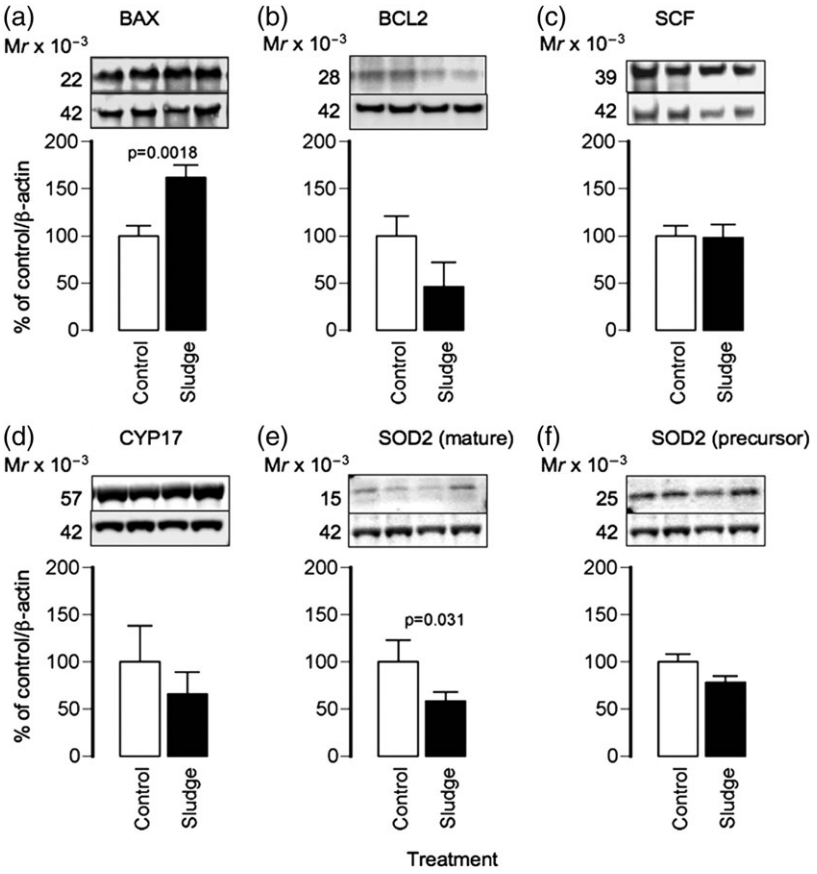

Figure 2: Quantification of levels of Day 110 fetal ovarian proteins ($n = 15$ control versus 8 treated ovaries) involved in (**a**) pro-apoptosis, BAX, (**b**) anti-apoptosis, BCL2, (**c**) developmental cell signalling, stem cell factor (SCF), (**d**) steroidogenesis, CYP17 and (**e** and **f**) oxidative stress response, manganese superoxide dismutase (SOD2).
P-values denote significant differences between control and sewage sludge-exposed fetuses and the load control was β-actin at 42 kDa.

this load control. *In utero* exposure to sewage sludge pasture altered the balance between pro- and anti-apoptotic processes in the fetal ovaries. Whereas BAX was significantly higher in treated ovaries relative to the controls (65%, $P = 0.002$), BCL2 tended to be lower but this difference did not reach statistical significance (54%, $P > 0.05$, Fig. 2a and b). Expression of the key ovarian developmental signalling protein, SCF, was not different between controls and sludge-exposed ovaries (Fig. 2c). CYP17 expression tended to be lower (34%) in the treated compared with the control ovaries, but again this difference did not reach statistical significance (Fig. 2d). SOD2 was detected as both the mature 15 kDa form (weakly) and the 25 kDa precursor form (Fig. 2e and f). The precursor form tended to be lower (22%, $P > 0.05$), but the mature molecule was significantly lower (by 42%) in the treated compared with the control ovaries ($P = 0.031$).

Effects of treatment on the fetal ovarian proteome

There was little difference in the overall number of robust protein spots between the control (349 ± 9) and sewage sludge (354 ± 5) groups ($P > 0.05$); however, 147 protein spots showed significant ($P < 0.05$) differences in normalized volume and presence or absence in the sewage sludge compared with controls, based on a minimum change of $\geq$1.2-fold, $P < 0.05$, for inclusion. The differentially expressed protein spots covered a wide range of normalized spot volumes. In the sewage sludge ovarian proteome, a total of 52 spots were up-regulated (1.2–4.6-fold increased, $P < 0.05$), 46 were down-regulated (1.2–5.2-fold decreased, $P < 0.05$), 25 were absent (only present in the control group) and 24 were unique (only present in the sewage sludge group). Nineteen protein spots showing consistent detection, reproducible quantification between replicate gels and statistically significant differences, or very clear uniqueness to one experimental group, were identified by tandem mass spectroscopy (Table II and Fig. 3). Six of these proteins were blood protein which showed highly variable alterations in expression (−5.2-fold to +3.5-fold, absent to unique) between sewage sludge and control groups, preventing any simplistic links to potential differences in vascularization. The remaining 13 proteins fell into four broad functional categories: (i) cytoskeleton and its regulation, (ii) gene expression, transcription and processing, (iii) protein synthesis and (iv) protein phosphorylation and receptor activity. Pathway Architect software (Stratagene Europe, Amsterdam, The Netherlands) was used to inform on the functional links between the proteins that were seen to exhibit altered expression between the two groups. Six of these proteins were directly linked with translation (Fig. 4a), and SOD2 and BAX are directly associated with DNA fragmentation during apoptosis (Fig. 4b). Graphical representation of an interactions network demonstrates that CALM1 dominates the relationships between many of the differentially expressed proteins (Fig. 5).

Discussion

The aim of this study was to establish whether long-term exposure of pregnant ewes to a cocktail of ECs disrupts female fetal ovarian development. Our results clearly show that long-term dietary exposure to

Table II. Positively identified proteins showing differential expression between ovaries at GD110 from 15 control fetuses with 8 fetuses exposed to sewage sludge *in utero*.

Spot #	Protein name *GENE SYMBOL*	Protein function and *Tissue/cellular location*	MW (kDa)	pI	MOWSE score	Accession number (NCBI)	Fold change (P, ANOVA)
Cytoskeleton and its regulation							
1	Gelsolin [actin depolymerising factor (ADF), brevin] *GSN*	Promotes nucleation and severs formed filaments. Expression lost in ovarian carcinoma. *Cytoplasm*	80.9	5.58	429	2833344	−2.8 ($P = 0.020$)
2	Vinculin *VCL*	Involved in cell adhesion and focal complex assembly with role in actin microfilament attachment to plasma membranes. *Cytoskeleton, cell–cell junctions.*	117.2	5.83	1050	4507877	−3.4 ($P = 0.043$)
9	Tubulin α-chain (alpha-tubulin 1) *TUBA1*	Major constituent of microtubules, binds two GTP molecules. *Cytoskeleton*	50.6	4.97	383	3502919	+3.1 ($P < 0.001$)
Blood proteins							
5	Serum albumin precursor *ALB*	Secreted protein, typically into plasma, binding molecule, regulates colloidal osmotic pressure	71.1	5.82	251	162648	−5.2 ($P = 0.003$)
10					97		+3.0 ($P < 0.001$)
12					189		Absent
8	Albumin precursor *ALB*		71.1	5.80	483	1387	+3.5 ($P = 0.010$)
16					646		Unique
14	Serotransferrin precursor (transferrin, siderophilin) *TF*	Iron-binding transport protein and stimulation of cell proliferation	79.9	6.75	243	29135265	−3.1 ($P = 0.003$)
Gene expression, transcription and processing							
3	DNA-binding pur alpha *PURA*	Interacts with RNA and DNA and recruits regulatory proteins to specific nucleic acid sequences, stimulating transcription. *Nucleus*	34.8	5.88	61	9652255	−5.0 ($P < 0.001$)
7	Heterogeneous nuclear ribonucleoprotein K (HNRPK) *HNRPK*	Binds RNA, interacts with pur alpha to mediate repression of CD43 promoter. *Ribonucleoprotein complex*	51.3	5.14	598	74354615	+3.1 ($P = 0.023$)
11	Heterogeneous nuclear ribonucleoprotein H (HNRNPH) *HNRPH1*	Part of complex providing substrates for processing of pre-mRNA. *Nucleus*	49.5	5.89	475	10946928	Absent
Protein synthesis							
4	Endoplasmic reticulum protein ERp29 (ERp31 or ERp28) *ERP29*	Molecular chaperone, participates in the folding of secretory proteins in endoplasmic reticulum. *Endoplasmic reticulum*	28.9	5.63	270	109658363	−3.2 ($P = 0.002$)
6	Calmodulin (CaM) *CALM1*	Mediates the control of enzymes and proteins (e.g. protein kinases and phosphatases) by Ca, role in calcium signalling. *Cytoplasm, cell membrane*	16.8	4.09	224	115509	−3.1 ($P = 0.033$)
17	Tu translation elongation factor (EF-Tu) *TUFM*	Role in protein synthesis, promoting GTP-dependent binding of tRNA to ribosomes. *Mitochondria*	49.7	6.72	235	111304949	Unique
18	Tumor rejection antigen 1 (GP96) (HSP90 family) *TRA1 (GRP94, HSP90B1)*	Molecular chaperone during processing, folding and transport of secreted proteins in the endoplasmic reticulum. *Endoplasmic reticulum*	92.7	4.76	727	27807263	Unique
Protein phosphorylation and receptor activity							
13	Guanine nucleotide-binding protein G, beta 1 (transductin beta 1 chain) *GNB1*	Part of G-protein heterotrimer, transduces transmembrane signalling systems. *Cell membrane, cytoplasm*	38.2	5.60	384	6680045	Absent
15	Protein phosphatase 1D *PPM1D (WIP1)*	Required for relief of p53-dependent checkpoint mediated cell cycle, negatively regulates cell proliferation. *Nucleus*	38.0	5.84	351	227436	+4.6 ($P = 0.010$)
19	S100 calcium-binding protein A11 (similar to) *S100A11*	Proposed function is calcium-ion binding and signal transduction, negative regulation of cell proliferation. *Cytoplasm*	11.5	6.72	202	29135265	Unique

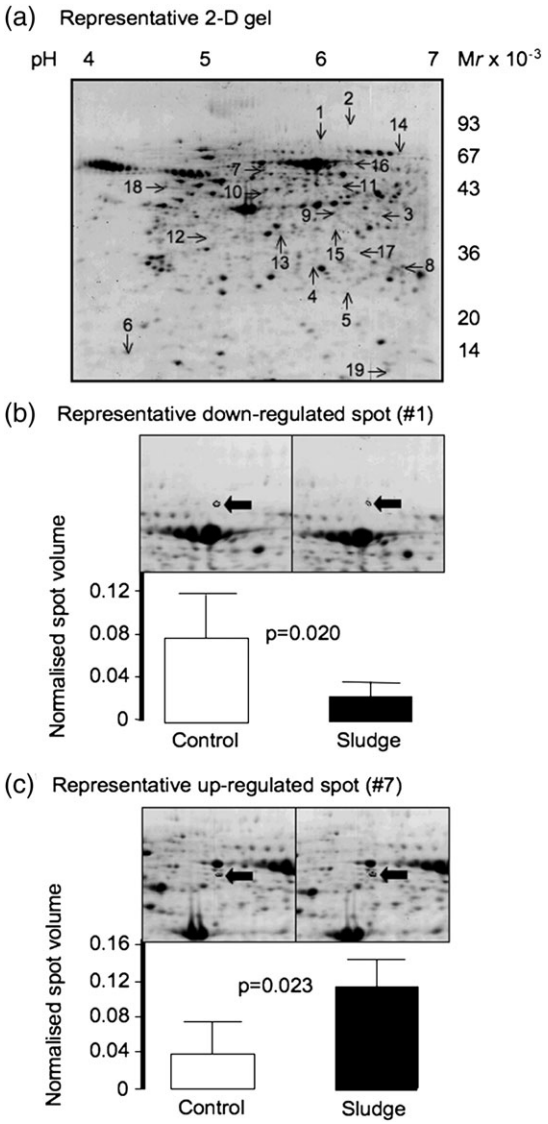

Figure 3: 2-DE gel electrophoresis based proteomic analysis of Day 110 fetal ovaries ($n = 15$ control versus 8 treated ovaries) pooled into treated and control groups.
(**a**) Representative 2-DE 7 cm gel using a 4–7 pH gradient. The numbered arrows show the location of the 19 protein spots identified in Table II. Zoom boxes demonstrating representative protein spots showing (**b**) down-regulation and (**c**) up-regulation in fetuses exposed to sewage sludge. The histograms are based on averaged ($n = 4$ gels/group) spot volume (normalized relative to total spot volumes separately for each gel). Significant differences are based on ANOVA following log-transformation of spot volumes.

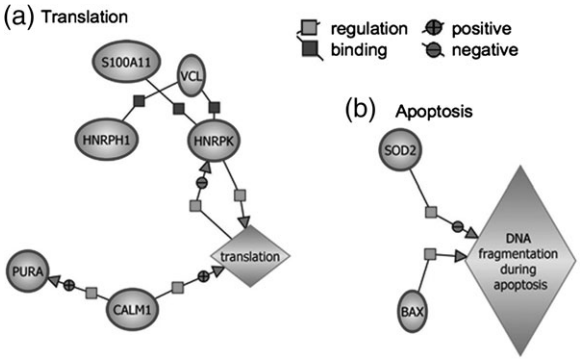

Figure 4: Biological processes network of some of the differentially expressed proteins from the treated ovaries, demonstrating key interactions between CALM1 and HNRPH in regulating (**a**) translation and antagonism between SOD2 and BAX in (**b**) DNA fragmentation during apoptosis.
Output from Pathway Architect.

such a cocktail, delivered via application of sewage sludge fertilizer to pasture, significantly disturbs both the fetal ovary and fetal endocrinology. These findings of alterations in the ovary were associated with a mild but significant growth restriction of the female fetuses.

In utero exposure of the female fetus to ECs results in a very wide range of morphological changes, varying according to species and nature of the chemicals (reviewed Miller *et al*., 2004). Although 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) does not produce the reduction in rodent oocyte and follicles numbers that we find (Flaws *et al*., 1997), PCBs and polycyclic aromatic hydrocarbons (PAHs) do (Flaws *et al*., 1997; Matikainen *et al*., 2002). The actions of other chemicals in the sewage sludge with effects on reproduction, such as cadmium (Henson and Chedrese, 2004), must also be taken into account. However, it is unlikely that our results were due to elevated phytoestrogens since studies of phytoestrogens (usually in rodents), such as genistein (e.g. Jefferson *et al*., 2006), show decreased germ cell apoptosis and reduced breakdown of oocyte nests, quite different from our findings of reduced oocyte density in fetuses exposed to sewage sludge chemicals *in utero*. This is extremely interesting given that ovarian BAX expression was significantly higher in sludge-exposed fetuses. In the mouse, BAX is a key regulator of follicle numbers and the BCL family is heavily implicated in the mechanisms underlying detrimental effects of selected environmental chemicals on the ovary (Borgeest *et al*., 2004; Miller *et al*., 2005; Greenfeld *et al*., 2007a,b). It is most probable that similar mechanisms act to determine the follicle pool in all mammals. That the anti-apoptotic BCL2 tended to be reduced, although not significantly so, in the sludge-exposed fetuses, suggests a redistribution of the pro- and anti-apoptotic balance more towards apoptosis. These findings agree with our observation that there were no differences in granulosa cell mitotic index and lead to the conclusion that the reduction in follicle numbers that we observed was due to an early loss of germ cells. Should these effects be reproduced in women exposed *in utero* to ECs as fetuses, it would be likely to result in reduced fertility and earlier onset of menopause. SCF was not affected by exposure to an EC cocktail in sewage sludge demonstrating the subtlety of the effects of a cocktail of chemicals. Clearly, while oocyte density is lower, the pre-granulosa or granulosa cells were producing the same amount of SCF, which is important in early follicle development (Parrott and Skinner, 2000; Jin *et al*., 2005). Sewage sludge exposure did not significantly affect the mitotic index (density of pH3-positive cells) of any ovarian cell type studied, other than endothelial cells. This suggests an effect on ovarian angiogenesis and supports our conclusion that chemicals in the sewage sludge are acting on the fetus since EDCs are know to have both stimulatory and inhibitory effects on angiogenesis (Tavolari *et al*., 2006).

Although neither was statistically significant, our findings of reduced levels of circulating estradiol and lowered ovarian expression of CYP17 in sludge-exposed fetuses were consistent with each other and suggest a mild impairment of steroidogenesis in these animals, possibly related to reduced germ cell proliferation (Pannetier *et al*., 2006) in sewage sludge-exposed fetuses. We quantified SOD2, because reactive oxygen species (ROS) induced by some ECs can damage developing organs (Ahmed *et al*., 2005). Further, methoxychlor and heavy metals decrease the expression of protective enzymes such as SOD1 and SOD2 in ovarian cells (Gupta *et al*., 2006; Nampoothiri *et al*., 2007), which would have developmental and steroidogenic consequences. Our animals exposed to a real-life

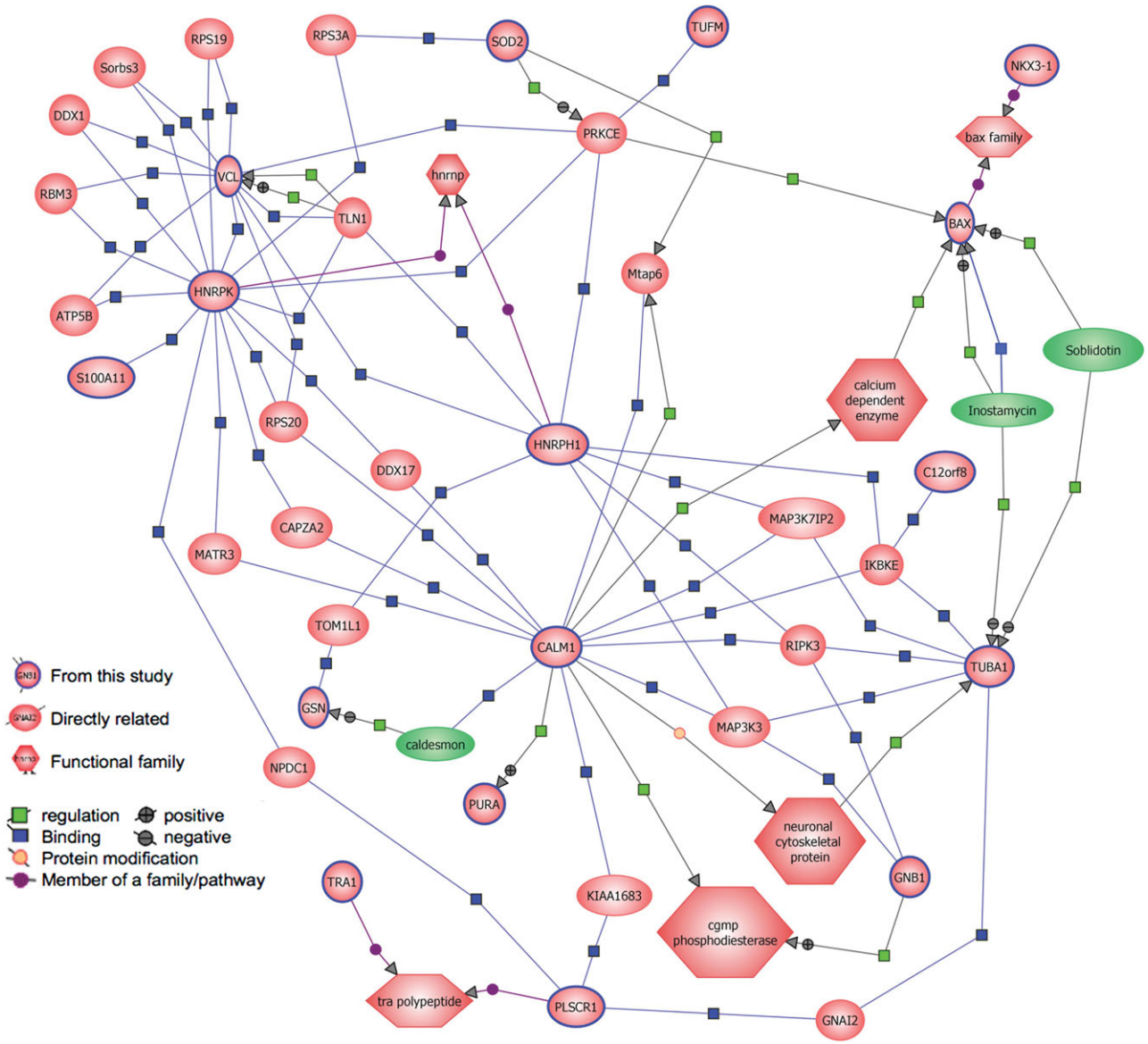

Figure 5: Biological interactions network of some of the differentially expressed proteins from the treated ovaries.
CALM1 sits central within the network, interacting with most of the differentially regulated proteins via one or two steps. Ovals with a green background denote small molecules. Output from Pathway Architect software (Stratagene Europe, Amsterdam, the Netherlands).

complex cocktail of ECs revealed a similar trend since the levels of SOD2 were lower than that in the controls and thus their ovarian cells may be more susceptible to oxidative damage than controls. The reduction in SOD2 would also imply reduced inhibition of DNA fragmentation during apoptosis at a time when increased BAX will elevate apoptosis rates (Fig. 4b; Southgate *et al*., 2006). The fact that phytoestrogens, such as daidzein, reduce ROS-induced toxicity (Tang *et al*., 2006), contrasts sharply with the effects of exposure to sewage sludge chemicals emphasizing that the observed effects are not due to phytoestrogens.

Exposure to sewage sludge chemicals altered the expression patterns of 42% of the protein spots in the exposed compared with control ovaries. Although this by no means reflects the entire ovarian proteome, it indicates that chemicals in sewage sludge can cause widespread changes in the ovary. Some of these changes will reflect altered cell numbers, but some will reflect changes in pathways involved in ovarian function and development. In addition, proteins may be present in many spots and spots may contain several proteins and so changes in spot volumes reflect complex and sometimes very subtle alterations in the protein profile. Proteomics allows us to investigate global effects of exposure to environmental chemicals without preconceptions about pathways and mechanisms, complementing the targeted study of ovarian morphology and proteins of known importance in reproductive development. We targeted the most consistently, statistically significantly, altered proteins for identification. This addresses gel–gel variation and reduces potential methodological error. In addition, we limited ourselves to proteins showing a $\geq$2-fold change in expression. Liquid-chromatography-mass spectrometry enabled positive identification of all 19 selected protein spots that fit the selection criteria. Overall, the proteins showing disturbed expression would be consistent with altered transcription and translation, apoptosis/proliferation and protein production/actions within the developing ovary. The disturbed proteins could also result in dysregulated receptor and calcium-dependent functions.

The most obvious consequence of the altered proteins is the probable disturbance in translation since 6 of the 19 differentially

expressed proteins, that we identified, have interactions and effects on translational processes (Fig. 4a). This would have consequences for the expression of gene products during development. The reduction of CALM1 protein in sludge-exposed fetal ovaries was unexpected because up-regulation of CALM1 is characteristic of genomic estrogenic activation (Wang *et al*., 2004). This, therefore, is evidence that exposure to sewage sludge was not necessarily activating conventional genomic estrogen pathways. Indeed, it is now well known that environmental chemicals operate through a wide range of mechanisms and pathways in addition to genomic estrogen action (Henley and Korach, 2006; Tabb and Blumberg, 2006). The 5-fold reduction of DNA-binding pur-alpha (PURA) in ovaries from fetuses exposed to sewage sludge chemicals is interesting on two counts. First, PURA, which recruits regulatory proteins to RNA and DNA sequences, is involved in oncogenic inhibitory pathways (Johnson *et al*., 2003), and, second, CALM1 stimulates PURA (Kuo *et al*., 1999), so a reduction in both proteins suggests a causative link. The location of CALM1 at the node of a biological interactions network that links most of the proteins we observed to be affected by exposure to sewage sludge chemicals (Fig. 5) demonstrates how changes in protein expression could propagate down numerous pathways involved from transcription along to post-translational modification and receptor activity. Relevantly, calmodulin-dependent kinases are implicated in maintaining ovarian steroidogenesis (Seals *et al*., 2004) and calmodulin binding protein is expressed in fetal germ cells during development (Luers *et al*., 2002), whereas defects in the caldesmon/calmodulin system impairs cell cycling and migration (Li *et al*., 2004). Gelsolin (GSN), 2.8-fold reduced in treated ovaries, was of interest since its expression is reduced in ovarian cancer (Noske *et al*., 2005). GSN, which modulates cellular motile activities, is widely expressed in the developing embryo (Arai and Kwiatkowski, 1999) and adult ovary (Teubner *et al*., 1994) where it is important in follicle growth. The role of vinculin (VCL) in a variety of processes, such as granulosa cell differentiation (Kranen *et al*., 1993), suggests that its down-regulation in our study may have consequences for ovarian development.

The HNRNP family of proteins is important in the formation of mRNA and both H and K forms are highly expressed in the primary oocyte (Kamma *et al*., 1995). HNRNPK, up-regulated in sludge-exposed ovaries, is important for cell spreading (Yoo *et al*., 2006) and transcriptional responses to DNA damage (Moumen *et al*., 2005). HNRNPK and PURA act together in transcriptional regulation of leukocytes (Da Silva *et al*., 2002) and, should both proteins act together on ovarian cells, disruption of transcription would be expected. Pertinently, the HNRNPH homologue, glorund, represses ovarian expression of *Drosophila* nanos (Kalifa *et al*., 2006), which is highly conserved and important for germ cell migration in mouse embryos (Tsuda *et al*., 2003). This contrasts with our study where HNRNPH was greatly reduced, which would suggest increased germ cell numbers. Disturbance of transcription would have multiple effects in many cell types, although redundancy of function could greatly reduce the severity of phenotypic effects.

The endoplasmic reticulum is important in protein secretion and many cellular activities, such as response to oxidative stress, so it is interesting that two endoplasmic reticulum chaperone proteins ERP29 and TRA1 (also called GRP94) were reduced and increased, respectively, by exposure to sewage sludge chemicals. Although not well understood, endoplasmic reticulum chaperone proteins are important in developmental and disease processes, including programmed cell death (Rao *et al*., 2006; Ni and Lee, 2007). TRA1 is abundant in the adult oolemma (Calvert *et al*., 2003) and regulates the secretion of a key player in ovarian function: insulin-like growth factor (Knight and Glister, 2006; Wanderling *et al*., 2007). TRA1 is increased in endoplasmic reticulum stress (Hagg *et al*., 2004) underlining the relevance of this finding. Although ERP29 is redox-inactive (Mkrtchian and Sandalova, 2006), it may be a target for reactive metabolites of toxic chemicals like bromobenzene (Koen and Hanzlik, 2002). The fact that pituitary levels of ERP29 are increased by exposure to estrogen (Blake *et al*., 2005), but decreased in our sewage sludge-exposed ovaries, may indicate either non-estrogenic effects or differential responses in the ovary. The increase in Tu translation elongation factor (TUFM), which has chaperone activities during protein synthesis (Suzuki *et al*., 2007), is interesting since mutations in elongation factors have developmental pathologies (Smeitink *et al*., 2006). Increased PPM1D and decreased GNB1 in the sludge-exposed ovaries imply effects on phosphorylation and receptor activity. PPM1D over-expression amplifies tumorigenesis by suppressing p53 activation (Bulavin *et al*., 2002) and enhances progesterone receptor activation (Proia *et al*., 2006). Importantly, progesterone receptor activation reduces primordial follicle assembly (Kezele and Skinner, 2003). This may be part of the mechanism by which sewage sludge-exposed ovaries exhibit reduced density of follicles at Day 110 of gestation.

The sludge-exposed female fetuses were 14% lighter than controls which raise the issue of what caused this difference and what effect this apparent very mild growth restriction might have on fetal ovarian development. There were no differences in maternal body weight in our study, which would contradict any conclusion that sludge-exposed fetuses were nutritionally challenged. The literature (reviewed Rhind *et al*., 2003) suggests that such a mild body weight difference is highly unlikely, in itself, to cause the ovarian developmental perturbation that we report here. For instance, a 50% maintenance diet during gestation resulted in a 32% decrease in lamb body weight and a 23% reduction in fetal ovary weight, but no loss of germ cells (Murdoch *et al*., 2003). More detailed studies of maternal undernutrition during pregnancy in sheep (e.g. Rae *et al*., 2001) found that cutting energy provision during pregnancy to 50% induced a trend towards smaller ovaries and significantly fewer more advanced follicles (types 2, 3 and 4) without changing germ cell numbers. This contrasts with our study in which the proportion of more advanced follicle increased, possibly contributing to our finding that ovary weight was not different between controls and sludge-exposed fetuses. Following growth-restricted fetuses into adulthood finds them recovering body mass with apparently normal hypothalamo-pituitary-ovary-axes, although ovulation rates were 20% reduced (Rae *et al*., 2002; Borwick *et al*., 2003). In the human, girls born small for gestational stage (i.e. growth-restricted) may have small ovaries and reduced ovulation rates at puberty (Ibanez *et al*., 2000, 2002), an effect that is at least partly related to insulin resistance (Ibanez and de Zegher, 2006), but probably not due to a reduction in the fetal follicle pool (de Bruin *et al*., 2001). The ovaries from our sludge-exposed fetuses are very different: no reduction in ovarian weight, but reduced germ cell and follicle densities and a small but significant increase in the proportion of more advanced follicles. Thus, we can conclude that growth restriction was not a major factor and, therefore, the effects of exposure to sewage sludge are much more likely to be due to chemicals in the sludge. That both the sewage sludge exposure in our study and the growth restriction of the sheep fetus (Phillips *et al*., 2001) have some similar reproductive consequences suggests some commonality in regulatory pathways between the two forms of fetal insult.

It is not clear which components of sewage sludge are reaching the maternal and fetal tissues and causing the disturbed fetal development we observed. It is currently impossible to accurately measure many chemicals in the fetal gonad because of the limited tissue quantity available. Therefore, we cannot be confident of actual fetal ovarian

exposure to different chemicals in our study. Some tissue EC levels do differ significantly between control and sludge-exposed fetuses (see Introduction), but it is most likely that subtle and complex alterations in the balance between a very wide range of chemicals, probably affecting a variety of body systems and organs, is the key factor in causing the disruption of fetal ovarian development. This is supported by our finding that although maternal smoking during gestation does not lead to any major changes in fetal liver PAH concentrations, the fetuses were correctly allocated to smoking and non-smoking groups by discriminant analysis of liver PAH concentrations (Fowler *et al*., 2008).

The sheep in our study were pastured on fields treated with sewage sludge in a way that may have raised exposure above the levels to which sheep are normally exposed to under current UK/EC legislation and agricultural guidelines; the treatments were designed to maximize the risk of exposure. It is important to note that our studies are designed to elucidate the effects of prolonged exposure to a real-life cocktail of chemicals and are not designed to determine whether the use of processed human sewage sludge as a fertilizer poses human health risks (under the current best operating practices). With this proviso, we can strongly conclude from our study that long-term exposure to low doses of a complex 'real-life' cocktail of environmental chemicals affects the developing fetal ovary, probably starting very early in fetal life, in such a way as to suggest that similar human fetal exposures could reduce fertility of the resulting women and increase their chances of premature menopause. Future studies should address genetic and endocrine mechanisms involved in these effects and also establish the critical windows of exposure, as well as determining the fertility of resulting adults.

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